Molecular Indicators of Chronic Stress in a Model Pinniped - The Northern Elephant Seal

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LONG-TERM GOALS

This effort seeks to identify combinations of biomarkers that are associated with discrete stress states in marine mammals. These taxa of concern are potentially affected by multiple environmental stressors. Some stressors may cause transient, acute responses whereas others may result in chronically activated stress responses with varying degrees of functional consequences. Differentiating among unstressed individiuals, those that have experienced recent acute stress, and chronically stressed animals can offer substantial information relevant to management and conservation of marine mammals potentially exposed to anthropogenic disturbance, including ocean noise.

OBJECTIVES

Limited data are currently available on molecular mechanisms that mediate the stress response in marine mammals. This insufficiency hinders discovery and development of markers of elevated stress and its consequences. Non-targeted -omics technologies offer a powerful approach for rapidly increasing our understanding of the molecular consequences of the stress response. Transcriptomics quantifies global changes in gene activity during a physiological perturbation; metabolomics quantifies global changes in metabolic products between distinct phenotypic states (Wang et al., 2009). Genes and metabolic pathways that are significantly altered between baseline and stressed conditions can be used as qualitative and quantitative markers of physiological stress. The objectives of the proposed effort are to determine molecular markers of stress using (1) transcriptomics and (2) metabolomics approaches, (3) integrate these molecular markers to discover commonalities, and (4) use these integrated markers to distinguish acute and chronic stress states in marine mammals.

APPROACH

Overview of Experimental Design

The proposed work will be conducted in northern elephant seals (*Mirounga angustirostris*), a tractable marine mammal system in studies of stress and its impacts (Champagne et al, 2012). Measurements will be conducted in juvenile elephant seals that reliably haul out each fall. This life-history stage represents a baseline state in this species with no additional confounding features such as breeding or molting. In each experimental group we will sample blood and blubber tissue. From blood, circulating levels of stress hormones will be measured and metabolomics analyses will be conducted; blubber tissue will be used for transcriptomics.

Experiment I - Establish ACTH dosing for chronic stress

To induce a chronic stress response, the HPA axis will be stimulated by repeatedly administering ACTH in juvenile seals. During previously funded work, we conducted ACTH administrations in several age classes of elephant seals, including juveniles, and determined the appropriate dose (0.25 U/kg) to elicit a reproducible acute stress response (Khudyakov et al 2015b). Repeated administrations will be necessary to induce a prolonged response. We will perform pilot experiments in five juvenile elephant seals to safely establish an ACTH administration regimen that consistently elicits a chronically elevated stress response. The dose and frequency of ACTH administration will be varied until circulating cortisol levels remain elevated for five days. This work will 1) establish the time course of a chronic stress response and 2) define "acute" and "chronic" time points for further sampling.

Experiment II - Chronic stress induction; Tasks 1, 2, & 3

Once an appropriate ACTH administration regimen is determined, we will conduct 5 chronic stress measurements in juvenile elephant seals. Blood and blubber samples will be repeatedly collected from study animals immediately before and periodically during HPA axis stimulation, as determined by Experiment I, to capture unstressed, acute, and chronic stress conditions (for example, immediately before and 2 hrs following the initial ACTH administration, and then 2 and 4 days later in the early and late phases of chronic stress).

Experiment III - Evaluating stress markers; Task 4

Stress markers determined in Tasks 1 and 2 will be evaluated in a separate study group. Juvenile northern elephant seals display variable cortisol concentrations at the onset of their fall haul-out, probably as a result of varying environmental stressors experienced during their previous foraging trip. Elevated cortisol concentration at the beginning of their haul-out indicates higher degrees of environmentally induced stress and has been linked with reduced foraging success (Crocker et al, 2011). We will use this natural variability in chronic stress to test gene and metabolite markers identified in Tasks 1 and 2. Blood and blubber samples will be collected from 30 study animals. We will evaluate HPA axis activity in these animals and subsequently select 10 subjects representing “low baseline stress” and 10 subjects representing “high baseline stress” conditions based on criteria from current projects (ONR project N000141110434). These 20 animals will then be used for stress marker evaluation in Task 4.

Task 1 - Gene expression changes in adipose during stress

Physiological processes are driven by gene products such as metabolic enzymes. A robust indicator of organismal response to a stimulus is an alteration in gene expression, measured as a change in RNA transcript abundance. Identifying specific transcripts upregulated during a physiological response can

elucidate genetic pathways driving that process. A highly sensitive method of quantifying global gene expression is RNA sequencing (RNA-seq; Wang et al., 2009, Khudyakov et al., 2015a). We propose to use this method to examine global gene expression changes in a readily accessible elephant seal tissue in response to an ACTH administration and to identify transcripts upregulated during stress. Blubber is a metabolically active tissue that responds rapidly to stress by releasing metabolic stores to meet increased energy demands, making it an optimal target in which to examine stress-induced molecular changes (Djurhuus et al, 2002). It is also frequently sampled in marine mammals and could therefore be a valuable tissue in which to assess stress states.

RNA isolated from blubber will be sequenced and the transcriptome will be assembled de novo using newly developed methods and a recently generated elephant seal muscle transcriptome (Khudyakov et al., 2015a & b). Differential expression analysis will be performed using a rigorous bioinformatics tool (edgeR) that utilizes a range of statistical methodology based on negative binomial distributions, including empirical Bayes estimation, exact tests, generalized linear models and quasi-likelihood tests, to identify transcripts with altered expression during stress conditions. During Task 1, transcriptomes from blubber collected at baseline, acute, and chronic stress sample points will be compared. These comparisons will provide insight on physiological differences between acute and chronic stress responses and the transition between stress states.

Task 2 - Profiling metabolite changes during stress

One of the principal roles of the stress response is to increase energy available in circulation to provide for immediate energy needs at the expense of energy reserves (Sapolsky, et al., 2000). This is facilitated by altering metabolic pathways (e.g. gluconeogenesis, lipolysis, and protein catabolism), resulting in changes in the circulating levels of metabolites, which respond rapidly to environmental stimuli. Marine mammals, however, may display altered responses to stress relative to terrestrial species resulting from the adaptations to living in a marine environment. For example, preliminary data from our lab suggest that during HPA axis stimulation northern elephant seals display an exaggerated lipolysis response (potentially a result of their large adipose stores) and a reduced amino acid release (potentially resulting from a protein sparing adaptation during fasting; Champagne et al., 2015). Numerous circulating compounds can be simultaneously identified using a broad-based metabolomics technique. Metabolomics yields the relative concentrations of hundreds of small metabolites from a single tissue sample . Resolving the biomarker profile of an animal following exposure to a stressor can provide a snapshot of hundreds of circulating metabolites liberated into the bloodstream during HPA axis activation, potentially illuminating myriad metabolic pathways involved in a stress response. We propose to evaluate the stress response in a marine mammal by assessing alterations in the metabolome. Metabolite markers associated with stress will be identified using ACTH administrations described in Experiment II. Metabolites that are consistently elevated during stress could then be used as markers of stress in free-ranging animals.

Task 3 - Integrating transcriptome and metabolome data

Evaluating the transcriptome and metabolome in Tasks 1 and 2 will provide substantial molecular information on the acute and chronic stress responses in northern elephant seals. Assessing both simultaneously and across varying stress states will provide an opportunity to integrate the transcriptional and metabolic responses to chronic stress. Novel systems biology computational methods will be developed and used to combine large data sets from multiple -omics sources. These methods will establish links between gene expression and metabolite responses and identify biological processes altered by perturbation by mapping altered components to metabolic pathways. To our knowledge this will be the first study to simultaneously assess both the transcriptome and metabolome

in any free-ranging marine mammal. By integrating the measurements in this way, we can discount uninformative components from each of the datasets and narrow the search for stress markers to those that influence whole-animal function.

Task 4 - Evaluating combined molecular markers of stress
We will evaluate the most informative stress markers, established in Task 3, in a separate study group. Seals with varying degrees of baseline stress will be sampled in Experiment 3. High initial cortisol in this group indicates a potential stress state resulting from environmental stressors over the previous months-long foraging effort. We will compare molecular stress markers among animals of varying stress states, including those exhibiting signs of chronic stress. Panels of stress-induced transcripts will be assayed using qPCR, while panels of circulating metabolites will be quantified by metabolomics along with traditional hormone markers. We have found that changes in gene expression quantified by transcriptomics methods can be reliably detected by qPCR in independent experiments (Khudyakov et al 2015b). This task will determine transcriptional and metabolic markers and their relative expression levels that are specifically associated with chronic stress in free-ranging marine mammals. These markers will provide a quantitative diagnostic panel potentially capable of differentiating among stress states.

Key Individuals and Collaborations
Transcriptome sequencing will be conducted in collaboration with the UC Davis Genome Center DNA Technologies Core in consultation with the Core Director, Lutz Froenicke, PhD (lfroenicke@ucdavis.edu). Transcriptomics processing and analysis and omics data integration will be performed in collaboration with the Lab for Intensive Data Biology, led by Professor Titus Brown, PhD (titus@idyll.org). Metabolomics sample processing is being conducted by *Metabolon, Inc*. and in consultation with the Science Development Director, Jeff Buckthal, PhD (JBuckthal@metabolon.com).

WORK COMPLETED

We are in the initial planning phase of this effort. We are currently preparing to conduct the pilot effort described in Experiment 1 to establish the ACTH dosing regimen, which will take place in October, 2015.

RESULTS

No results are yet available from this effort.

IMPACT/APPLICATIONS

The ability to estimate the effects of anthropogenic sound exposure on marine mammals directly affects the United States Navy's ability to comply with Federal regulations. This work will evaluate transcripts and metabolites upregulated in response to HPA axis stimulation to determine if these molecular markers are capable of distinguishing acute and chronic stress in marine mammals. This will be accomplished by evaluating expression of molecular stress markers such as described in this proposal in routinely sampled tissues in free-ranging marine mammals. The effort will be the first of its kind to characterize whole-animal and tissue-specific cellular responses during simulated stress conditions and evaluate potential molecular markers of chronic stress and its impacts. The product of this work will enable the Navy to more reliably detect deleterious, long-term stress and its impacts on marine mammals in accordance with National Research Council recommendations (2005).

RELATED PROJECTS

Variability of Hormonal Stress Markers and Stress Responses in a Large Cross-Sectional Sample of Elephant Seals; Grant #N000141110434. During this study, ACTH administrations were conducted to stimulate the HPA axis in northern elephant seals; these data provided the framework to conduct investigations of chronic stress proposed here. Additionally, baseline concentrations of relevant stress hormones in northern elephant seals across seasons and life-history stages were described and this extensive dataset will be used to determine low and high stress individuals in Task 4 of this effort.

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